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(54) Title: BIOACTIVE MOLECULE DELIVERY

(57) Abstract

A delivery vehicle for bioactive molecules such as antigens via an oral or injection route to present to the immune system the active molecule in a protected carrier which allows slow release of the molecules. Antigens are aggregated with cell fragments and encapsulated in a polysaccharide biofilm such as N-acetyl glucosamine. The encapsulated microparticles attach to a mucosal surface such as the gut lining and resist breakdown by gastric acid secretions. These microparticle delivery vehicles are useful for delivering oral vaccines for humans and animals.

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BIOACTIVE MOLECULE DELIVERY

This invention relates to delivery vehicles for bioactive molecules. In particular, it deals with the delivery of bioactive molecules via an injectible or oral route in a manner so as to present the active molecule to the immune system in a protected and slow release fashion thereby prolonging and enhancing the nature of the response generated.

BACKGROUND OF THE INVENTION

Traditionally, vaccines for preventing disease were delivered to a patient as an injection of an inactivated antigen derived from the causitive agent of interest. Such vaccines were thus disease specific and usually strain specific. Alternatively, live vaccines containing attenuated antigens have been delivered so as to provide a sub-clinical infection in the patient sufficient to generate an immune response. These vaccines rely on the mass delivery of the attenuated antigens, or the multiplication of the antigen in situ. One final alternative, killed vaccines, incorporate an antigenic portion of the causitive agent. This type of vaccine results in the stimulation of an immunoglobulin G (IgG) primary response.

Recently, beyond the use of vaccines for the treatment of a medical condition, attempts have been made to modify bodily function by the use of vaccines which are based upon peptides, with the intention that the peptides should act in an antigenic fashion, stimulating the production of antibodies which will effect physiologic function in the desired manner. One desirable alteration of physiological condition, particularly in the animal husbandry industry is the increase in the size of an animla intended for slaughter for human consumption, or alternatively, the reduction of fat content in animals intended for the same purpose. It was expected that for alterations of growth patterns in such animals the antibodies produced by peptide delivery in this fashion might be Immunoglobulin A (IgA) because of the recognised function of IgA in preventing establishment of infection by pathogens at gut level. These attempts to alter growth patterns in animals have, however, been disappointing, probably because immunogenic protection involves more than just antibodies to a particular peptide sequence.

There have been a number of attempts to orally deliver vaccines with the aim of delivering the antigen to the mucosal surface. These attempts have been directed at the mucosal surfaces because it has been expected that it would be at the mucosal surface of particularly the stomach that absorption of antigenic peptides might effectively occur. There are, however, two fundamental difficulties with oral administration of antigens: firstly, the very low pH environment of particularly the stomach results in destruction of a very high proportion of orally delivered antigen, and secondly, that proportion of antigen which does reach the mucosal surface does not necessarily stimulate a proportionate immune response.

One such attempt was made by Lee and Tzan (Antigenic Characteristics and Stability of Microencapsulated Mycoplasma Hyopneumonial Vaccine, Biotechnology and Bioengineering Vol. 40, pp 207-213 (1992)) who used the solvent evaporation method to microencapsulate Mycoplasma hyopneumoniae antigens with cellulose acetate phthalate. The cellulose acetate phthalate polymer is eroded by the alkaline environment of the small intestine. The authors record that the higher the polymer content, the slower the release rate of the antigen evidently because a longer period is required for complete degradation of the cellulose acetate phthalate enteric coating by the acidic environment.

Another attempt was proposed by Mowat et al (immune-stimulating complexes containing Quil A and protein antigen prime class I MHC - restricted T lymphocytes in vivo and are immunogenic by the oral route, Immunology 1991, 72, 317-372) who disclose the use of lipophilic immune-stimulating complexes (ISCOMS) which are cage-like micelles formed by spontaneous coalescence of phosphatidyl choline and cholesterol in the presence of Quil A (saponin).

Other alternatives include the use of gelatin capsules (Farag-Mahmod et al; Immunogenicity and efficacy of orally administered inactivated influenza virus vaccine in mice; Vaccine Vol. 6 June, 1988), liposomes and synthetic polymers.

A further disclosure of efforts in this area appears in Nishimura,K. et al (*Effect of multiporous microspheres derived from chitin and partially deacetylated chitin on the activation of mouse peritoneal macrophages. Vaciine, Vol 5, June 1987, pp136-140*). This paper describes immune stimulation following increased mouse peritoneal macrophage activity, i.e. an increase in circulating immune response. It is postulated that the chitin may behave in an adjuvant capacity. It has since been shown that there is a strong possibility however that peritoneal macrophages may not account for the immunostimulation from the delivery vehicles described in this article, and hence the function of the chitin with respect to this disclosure is unclear.

Patent literature in this area includes AU-A-15580/92 (Biotech Australia Pty Ltd) which discloses a delivery vehicle for transporting substances to the circulation or lymphatic drainage system of the host. This oral delivery vehicle comprises a microparticle coupled to a carrier so that the particle can be transported to the lymphatic drainage system or to the circulation via the mucosal surfaces. In this case, however, the carriers recommended are often toxic to the patient to whom the particle is to be delivered resulting in septic shock. Similarly, the Patent AU-B-594059 (Biotechnology Australia Pty Ltd) describes a complex comprising an immunogen linked to a carrier molecule. This arrangement also suffers problems of toxicity to the patient because of the carrier molecules recommended.

None of the above mechanisms are, however, effective at delivering antigens to elicit a sufficient antibody response at the desired site in the body such that a physiologic alteration of the animal being treated may occur.

Thus, it is an object of this invention to provide a delivery vehicle for bioactive molecules such that the bioactive molecule is able to generate an immunoglobulin or biological response in the subject to be treated with the possibility that bodily function is altered, and in particular to provide a delivery vehicle which delivers the bioactive molecule to an active site in as high a concentration as necessary and in a form suitable for passage through the mucosal lining. It is also an object to provide this delivery vehicle as both an

orally deliverable vehicle and alternatively, as an injectable vaccine, but preferably in the form of an oral vaccine.

SUMMARY OF THE INVENTION

To this end, there is provided a biodegradable microparticle comprising one or more bioactive molecules, and a biofilm polymer, said biofilm polymer being of a type such that transport of the bioactive molecule can be effected to the appropriate mucosal surface, and such that slow release of said one or more bioactive molecules is enabled, whereby an effective level of immune or biological response is generated.

The microparticle may further comprise a matrix element such that it is preferably of a size whereby on presentation to a mucosal surface, the microparticle is able to adhere and deliver the bioactive molecule to the active site. Depending on the size and nature of the bioactive molecule, it may itself comprise the matrix element of the microparticle.

This invention is partially predicated on the discovery that a suitably effective and sustainable immune or biological response can be stimulated using antigenic material contained within a delivery vehicle which is formed by a protective, structural substance, and preferably the co-aggregation of the antigenic material with other biological material (cells, secretions or extracts) to form artificial particulate biofilms which aid in the presentation of the antigen to the mucosal surface. Surprisingly, the immune response generated using such a delivery vehicle does not appear to be primarily IgG.

It is also partially predicated on the discovery that certain biofilm polymers can be used in the microparticle that are not affected by the pH of the environment into which they are introduced. Co-aggregation of the components of the microparticle may result from intra-, inter- or multigeneric cell to cell interaction. The co-aggregation enables the microparticle to be presented to a mucosal surface, such as the gut lumen, intact since the gastric acid secretions do not break down the biofilm polymer. Moreover the co-aggregation enables presentation of the bioactive molecule to the active site in a particulate size suitable for uptake.

It is also surprisingly found that vaccinations via the intraperitoneal or oral route, of inactivated microorganisms known to possess toxins in the cell wall when encapsulated in delivery systems according to the invention, do not result in endotoxic shock and subsequent poisoning of the subjects. Since vaccinations of unencapsulated organisms result in mortality, it is postulated that the biofilm polymer component of the delivery vehicles of the invention provides protection for the subject being treated against endotoxic shock by slow release of the toxin at levels low enough to avoid adverse affect, or by blocking receptor sites in the gut lumen.

Compared to prior attempts such as that disclosed in AU-B-594059, the delivery system of this invention addresses more than simply increasing distribution of the active agent by enabling it to pass into a cell. This invention addresses the functionality of the active agent when it enters the cell and improves the processing of the active agent within the cell not just the uptake as was the case with prior art attempts. It is the facilitation of processing within the cell that provides improved biological response so that bodily function is altered.

The biodegradable microparticles may be delivered orally, by injection, (subcutaneously, intramuscularly, intraperitoneally) intrarumenally, by eye drop, in utero or into an egg.

As bioactive molecules, antigens, bacterial or viral, are possible. In particular, whole virus, viral sub units, soluble protein, whole cells or cell fractions, or any combinations of these may be used. Also suited for co-aggregation into the microparticles of the invention are natural or synthetic peptides, particularly those having physiologic functions. Suitable examples here are somatotropin, somatotropin release inhibiting factor, gastrin, luteinising hormone releasing factor (LHRH), proteins associated with Alzheimers disease or diabetes, CCK (cholecystokinin), ACTH, corticotrophin releasing factor, diazepam binding inhibiting factor, endorphins, fibrinogen binding inhibitor peptides, gastrin inhibiting polypeptides, and gastrin releasing peptides. Of particular interest in this category of bioactive molecules is the peptide somatotropin release inhibiting factor which may be administered in the form of a microparticle so as to result in growth enhancement of the animal being

treated. It is also probable that naked DNA and plasmids could be delivered in the form of a microparticle according to the invention for the purposes of gene therapy.

Clearly, it will be appreciated that microparticles according to the invention could be targeted against a variety of body functions including brain function, heart and circulatory function, gastro-intestinal function, reproductive function as well as straight forward disease prevention. It is also envisaged that the microparticles of the invention will be able to passively deliver antibodies, drugs, vitamins or any combination of these or other bioactive molecules.

If the bioactive molecule is an organism or an antigenic part thereof, it need not be inactivated, although the advantage of inactivation is that a sub-clinical, immunogenic response in the patient does not occur.

Suitable methods of inactivation include irradiation, formalin, glutaraldehyde, betapropiolactone, heat or other suitable treatments. In some aspects of the invention it is preferred to inactivate viable organisms in an oily vehicle which preferably comprises triglycerides, canola oil, alkyl glycerol and lecithin. Antigens inactivated by this latter method preserve greatest antigenic intensity. The oily base containing the antigen may be converted into a water in oil emulsion by vigorous mixing in a suitable oil to water ratio such as 2:1. The oily base preferably comprises 6 parts triglycerides, 2 parts canola oil, 1 part alkyl glycerol and 1 part lecithin.

Passive antibodies may be prepared by first delivering inactivated antigens to chickens and then harvesting the antibodies from the yolks of eggs collected from the innoculated chickens. If necessary the bird may be administered a booster vaccination 2-3 weeks after the primary vaccination. Then inactivated antigens may be delivered in a stable water in oil emulsion, the oily base of which may comprise triglycerides, alkyl glycerols, vegetable oil and emulsifiers. Conventional adjuvants may also be added. The harvested yolk is diluted and the natural fatty substances in the yolk are precipitated out using propyl carboxyl methyl cellulose or similar materials. The water/ yolk solution is then concentrated by conventional methods and spray dried to yield

an antibody rich powder in which antibodies are coated with the biofilm polymer.

The biofilm polymers useful for the invention should not be effected by gastric acid secretions and should facilitate adherence of the microparticle to the mucosal surface in the case of an orally delivered vaccine.

Preferred biofilm polymers are those of a polysaccharide nature because of their frequent extracellular function especially in biofilm formation. For example, often polysaccharides form gels in water to create hydroskeletons augmenting the function of polysaccharides and manipulating the rate at which macromolecules move across cell membranes. These hydroskeleton type polysaccharides have been recognised as being involved in extracellular cell recognition phenomenon and thus are particularly well-suited for the purposes of this invention.

Because of its ability to make the polymer glue-like in promoting co-aggregation, and because of its protein binding and mucous membrane attachment properties, as well as its ability to shrink wrap co-aggregated materials, the peptido-glycan polymer of the polysaccharide N-acetyl glucosamine is particularly preferred for the purposes of the invention. It is commonly found in bacteria, fungi and invertebrates, where one primary function appears to be to provide rigidity to the organism or cell. The physical properties of the N-acetyl glucosamine base can be markedly altered by the addition of cations to the chemical structure and by complexing with other polymers. The N-acetyl glucosamine produces a flexible artificial cell wall for a construct which contains antigens. By this means an artificial particle can be produced having a diameter which is preferably regulated to less than 3 microns, thereby permitting targetting of the particle to uptake by the lymphoid system.

Another polymer group product found mainly in plant cells which may be useful constructing biofilms are the mannans. In nature they form part of the plant cell walls. More frequently mannans are found in nature as heteroglycans which produce polymers around the mannan base which replace or associate

with cellulose. They have properties ranging from structural to that of reserve carbohydrate sources as seen in the galactomannans.

A further polysaccharide polymer of interest is the xylans, and their close relatives the glucans. These polymers may be manipulated to have the properties of co-crystallising with microfibrils or providing hydrophobic coating for cellulosil structures.

Still other polysaccharide polymers are dextran (a high molecular weight storage polysaccharide - which is a glucan), agars, alginates and the glycosaminoglycans (hyaluronic acid, chondroitin, etc.)

A number of naturally occurring polysaccharide/protein complexes may also be especially suited to the purpose of the invention because of their recognised in vitro stimulation of an immunogenic response in cells of the lymphoid series.

For example, complexes from one mushroom species (genus plevfotus) which might be used include

a) protein containing xyloglucan with a polysaccharide - protein ratio of 76:24

b) protein containing mannogalactan with a polysaccharide - protein ratio of 76:16

c) protein containing xylan with a polysaccharide - protein ratio of 62:21

d) protein containing glucoxylan with a polysaccharide - protein ratio of 15:71

e) protein containing xyloglucan with a polysaccharide - protein ratio of 36:62.

In particular, it should be noted that the biofilm polymer component of the microparticle itself may also engender a non-specific immune response in the host which may act as a useful adjunct to the bioactive molecule itself present in the microparticle.

As the matrix component of the microparticle, one or more of whole cells, cell fragments, cell contents or purified protein from cells may be used because of their natural tendency to co-aggregate together, which tendency may be increased by the incorporation of biological extracts such as polysaccharides.

These matrix components may act as "cement" between the components of the biofilm microparticles, or may act as a network into which other components are incorporated. They are particularly useful because they enable manipulation of the size of the microparticle such that it may adhere and release the bioactive molecules through the mucosal lining. The pili of bacteria are particularly useful as the matrix component of the microparticle.

These matrix components may be bacterially or fungally derived.

The microparticles of the invention are preferably formed to a particle size between 0.7-10 micron in diameter. This size is the most opportune for presentation of particles to the mucosal lining if the particles are to be taken up.

Other materials which may be incorporated into the biofilm microparticles include phosphatides and fatty acids, such as mono- and triglycerides, both of which facilitate uptake of the bioactive molecule at the lymphoid cell by solubilising the surface membrane of lymphocytes, or additional polysaccharides to assist in countering the degradative effect of gastric acid secretions.

Adjuvants may also be added if the microparticles are to be delivered as an injectible. In particular, heat inactivated mycobacterium bovis cells or the peptide muramyl di peptide are suitable in this respect. Other currently available adjuvant bases which might be suitable include Freunds incomplete, Freunds complete or Quill A.

If the microparticles are to be orally delivered they may conveniently be enterically coated so as to minimise degradative effects of gastric acid secretions. This is not, however, essential. They may also be lyophilised prior to enteric coating so as to extend self life.

The microparticles of the invention are particularly advantageous for the following reasons:-

a) They are readily prepared.

b) They are safe to operators during manufacture and administration, and to the host following ingestion or injection.

c) The microparticles may be produced to any particle size desired.

d) The microparticles are attracted naturally to the mucous membrane lining of the gastrointestinal tract onto which they attach after oral administration, releasing antigen into the Peyers Patches.

e) The matrix of the microparticle is broken down by the cells of the host, producing metabolites which have a macrophage stimulating effect. This results in a non-specific stimulation of the immune response.

f) Microparticles are able to be coated after incorporation of the antigen with conventional enteric coating substances to provide protection against hydrolysis in the gastric juices of the stomach, or to be formulated appropriately for delivery via injection.

g) The microparticle matrix may take up any number of different antigens simultaneously if desired to induce immunity with concurrent protection against more than one antigen at a time.

h) If orally administered, adjuvant effects usually associated with the injection of live attenuated vaccines are avoided.

In another aspect of the invention there is provided a method of altering physiologic function comprising orally delivering a peptide in a manner whereby an immune response is stimulated at least partially blocking the activity of the delivered peptide and of equivalent naturally occurring peptide.

In a preferred aspect of this embodiment of the invention, there is provided a method of altering physiologic function comprising orally delivering a peptide in a manner whereby an immune response is stimulated at least partially blocking the activity of the delivered peptide and of equivalent naturally occurring peptide, said orally delivered peptide being encapsulated in a biodegradable microparticle comprising a biofilm polymer and preferably, a matrix element.

There is also provided in a more preferred embodiment of this aspect of the invention a method of growth enhancement comprising administering to a patient requiring such treatment an effective amount of a biodegradable microparticle, said microparticle comprising Somatotropin release inhibiting factor, a matrix element, and a biofilm polymer.

This embodiment of the invention could be particularly useful in the commercial farming of animals such as poultry, pigs etc. In particular, oral delivery of a growth enhancement vaccine is particularly cost effective and, in contrast to previous attempts wherein it has been necessary to deliver a vaccine by injection, this method is labour-saving.

There is provided in another aspect of the invention, a growth enhancement vaccine comprising a biodegradable microparticle according to the invention, said biodegradable microparticle comprising Somatotropin release inhibiting factor, a biofilm polymer and preferably, a matrix element in conjunction with suitable carriers and/or excipients selected according to the route of delivery of the vaccine.

In a further aspect of the invention there is provided a vaccine against neonatal diarrhoea comprising a biodegradable microparticle according to the invention, said biodegradable microparticle comprising the whole or an antigenic part of a live, attenuated or killed gut pathogen, a matrix element and a biofilm polymer in conjunction with carriers and/or excipients selected according to the route of delivery of the vaccine.

This aspect is particularly useful in the treatment of neonatal diarrhoea which is a very common syndrome of infant humans and mammals, especially piglets and calves. All of these species are frequently weaned early for social or animal husbandry reasons. Under these conditions the infant is deprived of the natural protection which comes from the mother via the secretory IgA in the milk. Secretory IgA is poured out at the epithelial surface of the mammary gland in response to antigen challenge to the dam. It is highly likely that the dam will encounter similar pathogens to the suckling neonate she feeds, thereby providing the infant with the antibodies required to fend off infection. Additionally, there is a very high quantity of IgG in colostrum. IgG is absorbed across the epithelial barrier of the neonate following ingestion. This absorbed IgG is the sole source of gamma globulin to the neonate. Clear evidence exists that the higher the level of circulating gamma globulin in neonates, the better equipped they are to repel the septicaemia conditions associated with diarrhoea. If neonatal diarrhoea is allowed to persist in infants, they rapidly

succumb to dehydration and/or septicaemia, rapidly weakening the animal, and death is common.

Suitable live, attenuated or killed gut pathogens for incorporation into the vaccines of this aspect of the invention may include:

Human: Rotavirus, Escherischia coli, Campylobacter spp

Calf: Rotavirus, Escherischia coli, Salmonella spp

Piglets: Rotavirus, Escherischia Coli.

There is also provided in this aspect of the invention, a method of treatment of neonatal diarrhoea comprising administering to a patient requiring such treatment, an effective amount of a biodegradable microparticle, said microparticle including the whole or an antigenic part of a live, attenuated or killed gut pathogen, a matrix element and a biofilm polymer.

In another aspect of the invention there is provided a method of forming a biodegradable microparticle comprising -

a) isolating the bioactive molecule,

b) forming the microparticle, and

c) forming said microparticle into a suitable delivery vehicle by incorporation of said bioactive molecule.

In a preferred form of this aspect of the invention, the method of forming a biodegradable aggregated biofilm polymer microparticle comprises:

a) growing up an antigen in appropriate facilities (eg in embryonating hen eggs),

b) harvesting, washing and concentrating by centrifugation said antigen and preferably storing it at about 4°C in phosphate buffered saline, or egg yolk citrate buffer solution,

c) inactivating said antigen by the method most appropriate to that antigen,

d) preparing a saline solution into which is dissolved a cation-manipulated derivative of suitable biofilm polymer. This solution preferably is kept stirred,

e) adding a calcium chloride solution to the preferably stirred solution, which is conveniently chilled to 4°C.

f) adding small quantities of suitable metal ions into the solution and stirring until the solution gels,

g) sonnicating and centrifuging said gel and then washing the sedimented microparticles,

h) resuspending the microparticles in saline and stirring, then adding concentrated antigen slowly,

i) centrifuging and either air drying, or drying the microparticles in a low temperature fluid bed dryer to shrink the biofilm if necessary.

j) coating the dried microparticles if necessary with conventional gastric protective films (eg solvent based cellulose acetate phthalate), or incorporating them into tablets or capsules which may then be coated to protect against gastric hydrolysis for oral administration.

If the microparticles are to be used in an injected preparation they may be suspended in suitable vegetable oil carriers.

Because the biodegradable aggregated biofilm polymer microparticle is intended to be administered to the patient by ingestion, or injection, they may be shrunk to provide for

a) removal of moisture thereby prolonging storage shelf life of the antigen

b) reducing particle size of the cells to optimise biological uptake by target cells if necessary.

In an alternative method of producing biodegradable microparticles, the microparticle is formed by polymerisation directly around the bioactive molecule. The microparticles may then be harvested and shrunk if necessary, and if desired enterically coated prior to use.

Preferably, this alternative method of producing a simpler biofilm microparticle comprises harvesting the antigen (virus) into an egg yolk citrate buffer solution, concentrating in this medium by centrifugation, washing it and placing it into a saline solution to which is added the manipulated biofilm polymer which is then polymerised by the addition of calcium and any other desired heavy metal cation. The beads produced may then be harvested and shrunk by drying and enteric coated (if desired) prior to use. Inactivation of the

antigen occurs ideally after concentration from the egg yolk citrate buffer solution.

It will be appreciated that significant variations may be contemplated to the above-mentioned methods to cater for special bioactive molecules and specific uptake requirements such as if vitamins are contemplated as the bioactive molecules.

DESCRIPTION OF THE INVENTION

The following examples serve to illustrate the invention. It will be appreciated that these examples do not represent the totality of the microparticle types possible.

Example 1 - Production of Microparticles using N-acetyl glucosamine

N-acetyl glucosamine in the form of cationic Beta 1,4 linked glucoses possessing charged amino groups (chitin) is the starting point for the particles. Partial deacetylation of this material is performed in the presence of keto acids according to published methods. This reaction occurs in the presence of trace quantities of calcium chloride and sodium dihydrogen orthophosphate in the ratio of 98 parts of polymer, and 1 part each of the mineral salts. Dehydrating this material results in a chelated acetyl glucosamine polymer.

This material is then dissolved (2 gm) in 100 ml of a 2% acetic acid solution, and hydrated for 24 hours by gentle stirring. The resultant solution is slightly viscous. If preformed granules are desired, additional calcium chloride is added in an alkaline solution while stirring. When pH of the solution reaches 7 the acetyl glucosamine precipitates out as floccules. These may be spun down by centrifugation, and washed in phosphate buffered saline. Diameter of the granules is regulated by the speed of addition of the alkaline solution - large granules form with rapid addition, while fine particles result after very slow addition.

If, however, it is desired to produce a preshrunk granule which contains an antigen, the antigen and preferably its carrier (eg cell wall fragments, pilli etc) are first complexed together by suitable linking agent (eg gluteraldehyde). The dilute acetic acid solution containing the polymer is then gently added to that

containing the linked antigen-carrier complex, and gently stirred. Alkaline calcium chloride solution is then gently added dropwise while stirring until pH approaches 7, at which point the solution yields a white haze, which is the formation of the microparticles. Purification of the particles occurs from washing in phosphate buffered saline and centrifugation. The microparticles may be airdried and shrunk prior to use as a vaccine.

Example 2 -Bacteria + Virus + Peptidoglycan Polymer Microparticles

Influenza virus was grown in embryonating hen eggs. Infected allantoic fluid was harvested and inactivated by gamma irradiation.

Haemophilus influenza organisms were grown up on blood agar plates, harvested into phosphate buffer solution and inactivated with 0.15% formalin for 1 hour.

The inactivated influenza virus was suspended in phosphate buffered saline ($10^6 EDI_{50}$ per ml) was added to the inactivated H influenza (in phosphate buffered saline (10^9 bacteria per ml) and allowed to co-aggregate in a rotator for 30 minutes. To this mixture of organisms was added an equal volume of a solution of 5% N-acetylglucosamine in the form of Chitosan-F in 7% lactic acid (aqueous base). Mixing continued for a further 30 minutes. Calcium chloride solution was then dripped into the agitated mixture until gelling occurred. The gelled product was then sonnicated for 5 minutes until fine beads formed. These beads were then washed and dried at 4°C.

The beads were then administered orally to 6 week old mice (previously unexposed to influenza virus) in a 2ml dose of 2%$NaHCO_3$/gelatin solution (for protection against gastric acidity) each day for 3 days. Fourteen days after the last immunisation the mice were killed and lungs and serum collected for antibody assessments to influenza virus using the Elisa test. Control mice were orally dosed with PBS on immunisation days.

Results were:

	Vaccinates		Control	
Mouse	Eli/ml lung	E l 1 / m l serum	Eli/ml lung	E l 1 / m l serum
1	70	52	0	0
2	62	37	0	0
3	23	49	0	0
4	18	28	0	0
5	35	37	0	0
Mean	41.6± 20	40.6 ± 11	0	0

Example 3 - Virus + Cell Wall Fragment + Peptidoglycan Polymer Biofilm Microparticle

E. Coli organisms were grown up in broth. The organisms were inactivated by the addition of 0.15% formalin to the broth and mixing for 1 hour.

Pilli of these cells were recovered from the organisms.

Newcastle disease virus was grown in embryonating hen eggs. Infected allantoic sac contents were recovered and treated with betapropiolactone to inactivate.

Washed pilli were mixed with washed inactivated Newcastle disease in an egg yolk glyceral buffer solution and agitated for 30 minutes at 40°C. To this was added approximately an equal volume of preformed beads of N-acetylglucosamine. Agitation continued for a further 30 minutes.

The beads were recovered, washed and shrunk by drying at 4°C.

These shrunk beads were reconstituted into phosphate buffered saline solution and dosed in 2ml of a 2% $NaHCO_3$/ gelatin buffer to protect against degradation, to 6 weeks old male mice daily for 3 days. Two weeks after the last dose the mice were sacrificed and blood was collected for measurement of circulating antibodies to the ND virus (by Elisa test).

Results were

Mouse	Control Serum EL1/ml	Vaccinates Serum EL 1/ml
1	0	92
2	0	78
3	0	81
4	0	65
5	0	72
Mean	0	77.6± 5.2

Example 4 - Virus + Phospholipid + Peptidoglycan Biofilm Microparticle

Influenza virus was grown up in embryonating hen eggs. Allantoic fluid containing the virus was harvested and subjected to gamma irradiation to inactivate the virus.

Inactivated virus was washed in phosphate buffered saline solution. Virus was then homogenised in a phosphate buffered saline solution containing 10% egg yolk lecithin. To this was added an equal volume of 5% N-acetylglucosamine in 7% lactic acid (aqueous solution). The solution was agitated for 1 hour at 4°C. To this solution was added by trickle feed a calcium chloride/cellulose acetate solution, agitating all the while the addition took place. Once gelling occurred addition ceased. The gel was then sonnicated until beads formed. The beads were washed in deionised water and shrunk by drying at 4°C.

Beads were reconstituted in a 2% $NaHCO_3$/Gelatin solution for vaccination orally each day for 3 days to six weeks old mice never previously exposed to influenza virus.

Two weeks after the last vaccination the mice were sacrificed and blood was collected to measure serum antibody levels to the influenza virus using the Elisa test.

Results were

Mouse	Control EL1/ml	Vaccinates EL1/ml
1	0	47
2	0	68
3	0	52
4	0	39
5	0	47
Mean	0	50.6 ± 7.1

In a further illustration of the scope of the invention microparticles containing virus were also produced by aggregating virus particles, phosphatidyl choline and a peptidoglycan. The gel produced was reduced to shrink beads which also were able to induce circulating antibodies to the virus antigen following oral administration.

Example 5 - Assessment of Immunogenicity of Microparticle Formulations According to the Invention

Vaccine efficacy is improved by increasing the immunogenicity of antigens. For systemic administration, but more particularly for mucosal administration, particulate antigens are generally more immunogenic than soluble antigens. While antibody production following vaccination may be protective for vaccines directed against mucosal viral diseases (eg influenza), as previously mentioned it is essential that, in addition to antibody production, there is effective cell-mediated (T cell-dependent) effector activities (eg cytotoxicity). This is particularly important with protein or subunit antigens which usually provoke poor cell-mediated responses.

The preshrunk biopolymer microparticle according to the invention has been developed to enhance immunogenicty of poorly immunogenic antigens and is particularly well suited for oral delivery. Experiments are reported here which demonstrate the efficacy of the formulation for both antibody production and cell-mediated immunity following both systemic and mucosal delivery.

Example 5A - Assessment of Immunogenicity of Microparticle Formulations for T Cell Activation

For vaccines directed against mucosal viral diseases (eg influenza) it is essential that, in addition to antibody production, there is effective cell-mediated (T cell-dependent) effector activities (eg cytotoxicity). This is particularly important with protein or subunit antigens which usually provoke poor cell-mediated responses. Here the T cell responses to OVA (ovalbumin) protein as a model antigen have been assessed using an *in vitro* cytokine release assay.

This example shows an evaluation of the efficacy of microparticle formulations according to the invention containing OVA in stimulating T cell activation.

T cell activation was determined by co-culturing an antigen-presenting cell line (LB cells) with an OVA-specific T cell line (Clone 1.1) in the presence of test antigen. IL-2 production, a measure of T cell activation, was then determined by adding serial dilutions of culture supernatants to secondary cultures of an IL-2-dependent T cell line (CTLL cells).

Cell Lines

LB	Antigen-presenting cells
Clone 1.1	OVA-specific T cell line (OVA-specific TcR)
CTLL	IL-2-dependent indicator T cell line

Antigens

OVA	Native ovalbumin (Sigma Grade V)
OVA-Nac	Ovalbumin incorporated in Inovax N-acetyl glucosamine microparticles
OVA-Nac-p	Ovalbumin conjugated to *E.coli* pili prior to incorporation in N-acetyl glucosamine microparticles according to the invention.

Pili conjugation (OVA-p)

Preparation of pili suspension

E. coli organisms grown in nutrient broth yeast extract 1% at 37°C for 24 hours. Culture concentrated to 50 ml by centrifugation and held at 4°C. Suspension then washed in PBS, resuspended to 10^9 organisms/ml, then

placed in a water bath at 60° for 30 minutes. Cell debris removed by centrifugation at 12000 G for 30 minutes (pili remain in supernatant).

50 mg OVA added to 5 ml pili solution (See Protocol #001 for method), stirred at room temperature for 10 min then 50 µl 25% glutaraldehyde added. The conjugate was stirred at room temperature for a further 30 min.

Microparticle preparation

5 ml OVA solution (10 mg/ml) or 5 ml OVA-p stirred for 10 min with 0.5 ml 2% N-acetyl glucosamine in glacial acetic acid, 0.5 ml 2.5 M $CaCl_2$ and 1 M NaOH (dropwise) until suspension formed.

Cell Cultures

LB antigen-presenting cells and Clone 1.1 OVA-specific T cells were cultured in the presence of 100 µl of serial dilutions of each of the antigen preparations for 24 hours. Culture supernatnats were then aspirated and added to secondary cultures of the CTLL IL-2 dependent cell. The proliferatin rate of these cells, which is proportional to the amount of IL-2 in the added culture supernatants, was detected by ^{3}H-thymidine incorporation measured as radioactivity counts per minute (cpm) in cells recovered from each culture. (See Figure 1).

Example 5B - Immunogenicity of Microparticle Formulations Using the LB Antigen-Presenting Cell Line

This example evaluates the efficacy of microparticle formulations containing OVA in stimulating T cell activation using the LB antigen-presenting cell line.

Cells derived from the LB antigen-presenting cell line and Clone 1.1 (OVA-specific T cells) were cultured in the presence of 100 µl of serial dilutions of each of the antigen preparations for 24 hours. Culture supernatants were then aspirated and added to secondary cultures of the CTLL IL-2 dependent cell line and IL-2 production determined as described above.

The results in Figure 2 show that the OVA-Nac preparation was superior to native OVA in stimulating antigen-specific T cell activation. Although the

OVA-Nac-p preparation was superior to native OVA it did not produce a response as great as that with OVA-Nac.

Example 5C - Immunogenicity of Microparticle Formulations Using the LB Antigen-Presenting Cell Line - Repeat of Experiment A with Additional Controls

This example repeats Experiment A and validates the assay by incorporating additional controls.

Cells derived from the LB antigen-presenting cell line and Clone 1.1 (OVA-specific T cells) were cultured in the presence of 100 µl of serial dilutions of each of the antigen preparations for 24 hours. Culture supernatants were then aspirated and added to secondary cultures of the CTLL IL-2 dependent cell line and IL-2 production determined as described above. Control cultures were also established in which antigen-presenting cells or T cells were omitted.

Results

The results in Figures 3-5 show that, as in Example 1, the OVA-Nac preparation was superior to native OVA in stimulating antigen-specific T cell activation, but the OVA-Nac-p did not produce a response as great as that with OVA-Nac. The lack of response in cultures of antigen-presenting cells without T cells or without antigen-presenting cells validates that the assay is measuring T cell reactivity to biologically processed antigen, as would occur *in vivo*.

Example 5D - Immunogenicity of Microparticle Formulations Using Splenic Antigen-Presenting Cells

This example evaluates the efficacy of microparticle formulations containing OVA in stimulating T cell activation using antigen-presenting cells derived from fresh murine spleen.

Antigen-presenting cells were prepared from freshly isolated murine spleen by trisammonium chloride lysis of erythrocytes followed by 300 rad γ-irradiation. These were co-cultured with Clone 1.1 (OVA-specific T cells) in the presence of 100 µl of serial dilutions of each of the antigen preparations for 24 hours. Culture supernatants were then aspirated and added to secondary cultures of the CTLL IL-2 dependent cell line and IL-2 production determined as

described above. Control cultures were also established in which antigen-presenting cells or T cells.

The results in Figure 6 show that, as in Examples 5B and 5C the OVA-Nac preparation was superior to native OVA in stimulating antigen-specific T cell activation and the OVA-Nac-p did not produce a response as great as that with OVA-Nac. These data demonstrate that the improved efficacy of the Nac microparticle formulation is not an artefact of the antigen-presenting cell line chosen for Examples 5B and 5C and provides further evidence that the assay is measuring T cell reactivity to biologically processed antigen, as would occur *in vivo*. These data also confirm that, at least in this assay system, pili are not essential to produce a desirable response.

Example 6 - Immunogenicity of Microparticle Formulations for Delivery of Viral Antigens assessed In Vivo

This example determined in mice the efficacy of a vaccine comprising inactivated influenza virus prepared in microparticles according to the invention, (Flu-Nac) presented as a biodegradable water-in-oil emulstion for intraperitoneal (IP) injection or as an aqueous suspension for oral/intranasal (IN) administration.

Influenza virus (NSW / Duck / Ukraine H1N8) was inactivated by γ-irradiation and incorporated into Inovax Nac microparticles as described in Example 1. This was delievered either in saline or as a biodegradable oil-in-water emulsion. The emulsion was prepared by combining microparticles in aqueous phase with radiamulus 2108 in equal proportions with 0.25 mg/ml *Mycobacterium bovis* and 13% w/v lecithin.

Mice were allocated to each of the following treatment groups:

GROUP	Day 0	Day 14	Day 21
Group A	IP Flu-Nac in oil base	ORAL + IN Flu-Nac in aqueous base	Assay
Group B	IP Flu-Nac in oil base	IN Flu-Nac in aqueous base	Assay
Group C	IN Flu-Nac in aqueous base	–	Assay
Group D	ORAL Flu-Nac in aqueous base	–	Assay
Group E	Unvaccinated controls	–	Assay

Mice were sacrificed at day 21 and serum and bronchial lavage collected. Lung, small intestine and spleen tissues were collected for fluorescent immunohistology and the remainder of the lung homogenised for antibody analysis. Bronchial lavage and serum samples were subjected to ELISA assay to determine presence of antibodies to influenza whole virus. Tissues will be prepared for double fluorochrome immunofluorescence to detect antibody-containing cells directed against influenza haemagglutinin.

The results from antibody determinations (shown in Table 1 - see over) indicate that in animals receiving prior priming (by the IP route) a substantial increase in both systemic (serum) and local (bronchial) antibodies was observed in mice given vaccine by the oral or intranasal routes. It is not known whether the IP injection is causing systemic or mucosal priming since previous studies have demonstrated that IP delivery of antigen in appropriate oil-based formulations can result in both systemic and mucosal priming (via the intestinal serosal surface). A single intranasal or oral administration in unprimed mice produced a small response in some animals.

TABLE 1

ANTIBODY RESPONSE IN MICE IMMUNISED WITH INFLUENZA VIRUS IN INOVAX CARRIER FORMULATION (% OF POSITIVE STANDARD)

ROUTE:	IP + ORAL/INTRANASAL		IP + INTRANASAL		INTRANASAL		ORAL		CONTROLS	
	BRONCHIAL LAVAGE	SERUM	BRONCHIAL LAVAGE	SERUM	BRONCHIAL LAVAGE	SERUM	BRONCHIAL LAVAGE	SERUM	BRONCHIAL LAVAGE	SERUM
	20.08	13.87	60.77	26.27	10.70	11.50	6.43	11.53	9.92	9.08
	16.84	12.81	21.15	20.31	3.81	11.01	6.15	10.40	6.47	6.56
	10.65	12.14	19.22	18.20	3.28	10.89	3.24	9.76	5.57	6.11
	NA	10.18	17.54	17.19	1.93	7.35	2.01	6.63	4.22	6.03
	6.27	9.57	14.30	17.04	1.56	5.88	1.23	5.84	3.57	5.69
	5.70	8.52	13.97	15.60					1.43	5.58
	4.92	8.22	NA	15.26					NA	4.98
	4.88	5.99	NA	13.85					NA	4.67
			5.78	12.63						
			5.41	10.55						
			4.71	10.33						
ALL ANIMALS:										
MEAN	9.91	10.16	18.09	16.12	4.25	9.32	3.81	8.83	5.20	6.09
STD DEV	6.24	2.64	17.16	4.58	3.72	2.54	2.38	2.47	2.89	1.36
RESPONDERS:										
MEAN	18.46	12.25	24.49	17.38	10.70	11.50	6.29	10.97	NA	NA
STD DEV	2.29	1.56	17.99	4.04	NA	NA	0.20	0.80	NA	NA

= NON-RESPONDERS (DEFINED AS LAVAGE VALUE ~≤ CONTROLS)

Example 7 - Immunogenicity of Microparticle Formulations for Delivery of Bacterial Subunit Antigens Assessed In Vivo

This example determined in chickens the efficacy of a vaccine comprising a *Campylobacter jejuni* flagellin subunt antigen in microparticles according to the invention (Flag-Nac) presented as a biodegradable water-in-oil emulsion for intraperitoneal (IP) injection or as an aqueous suspension for oral/ontranasal (IN) administration.

The antibody response in serum, bile and intestinal secretions was measured following immunisation of 3 week old birds with preparations containing C. *Jejuni* flagellin protein (Flag). Immunisation formulations containing microparticles according to the invention were compared with control groups either not immunised or immunised with Flag in a non-biodegradable oil-based adjuvant (montanite) which had been shown in previous studies to stimulate antibody production in chickens but to cause significant inflammation.

Birds were immunised at 21 days of age, then again at 36 days of age. Birds immunised orally received antigen for 3 successive days from 21 days of age (Gp. D) and 36 days of age (Gps. C and D). Birds were immunised in groups of 10 birds according to the following schedule:

Group A: Unimmunised controls

Group B: Intraperitoneal (IP) Flag-Nac in Inovax biodegradable oil base at 21 days, IP repeat at 36 days.

Group C: IP Flag-Nac in biodegradable oil base at 21 days, ORAL Flag-Nac in saline at 36, 37 and 38 days.

Group D: ORAL Flag-Nac at 21, 22 and 23 days, ORAL repeat at 36, 37 and 38 days.

Group E: IP Flag-Nac in inflammatory oil-based adjuvant at 21 days IP repeat at 36 days.

Birds were euthanased 2 weeks after the last immunisation, and serum, bile and intestinal contents collected and stored for antibody analysis. Antibody specific for Flag protein was measured by enzyme linked immunosorbent assay (ELISA), at a final dilution of 1/50 for serum, 1/40 for bile and 1/12 for gut

secretions. Results are expressed as OD%, calculated as the OD value in ELISA relative to a positive standard serum.

The antibody titres in serum, bile and intestinal contents are presented in Figure 7A, B and C. Whereas the inflammatory oil-based adjuvant produced the highest IgG response in serum, the Inovax preparation was equal or superior in promoting an IgA and IgM responses in serum and bile. Antibody in gut secretions was present at only very low levels, with significant titres only for the inflammatory oil-based adjuvant for IgG and for the Inovax microparticles given IP for IgA.

In vitro studies demonstrate that T cells cultured in the presence of the microparticle preparation according to the invention produce significantly higher levels of IL-2 than those cultured with native antigen. The fall in IL-2 activity of supernatants from OVA-Nac-p preparations at high concentrations and the high variability of these data may have been due to carry-over of lipopolysacchardie (LPS) from the pili in this antigen preparation, since the CTLL indicator cells are sensitive to the toxic effects of LPS. The data imply that the formulations without pili are superior, at least in this assay system, in enhancing antigen presentation and processing leading to superior T cell activation. For immune responses where cell mediated reactions are important, such as those directed against viral infections, the microparticles according to the invention show great promise as a delivery vehicle. Studies in Examples 6 and 7 demonstrate the potential for this formulation to produce an effective antibody response using either a viral antigen or a bacterial subunit antigen *in vivo*.

Example 8 - An Injectible System Delivering Somatotropin Release Inhibiting Factor To Enhance Growth

This example illustrates the suitability of the delivery system of the invention for the purposes of delivering bioactive molecules which result in an enhancing of the growth of the subject to which the vaccine is administered.

This particular example of the invention is directed to the subcutaneous or intraperitoneal delivery of somatotropin release inhibiting factor to chickens so as to enhance their growth.

Production of Solution A

A 1% nutrient broth yeast extract is prepared, innoculated with E. coli and incubated for 24 hours at 37°C. The bacterial cells are then concentrated about 20 times by centrifugation. Washing by centrifugation and discarding of the supernatant is performed three times using phosphate buffered saline (pH7.2) as the vehicle. The final washed suspension of cells will contain about 10×10^9 cells per ml.

The washed cells suspended in phosphate buffered saline are then placed in a water bath at 60°C and held there for 30 minutes. This process separates the pilli from the cells. The cell debris at the bottom of the centrifuge tube is discarded. The pilli in the supernatant are diluted and held at 4°C until further processed.

Somatotropin release inhibiting factor (SRIF) is solubilised in phosphate buffered saline, which is then mixed with the pilli suspension at a rate of 1mg of SRIF to the pilli recovered from the 10^8 E. coli cells and agitated for 10 minutes.

The pilli and SRIF are then combined together by adding 0.1 ml of 25% glutaraldehyde solution per 10ml of solution and agitating for 30 minutes. To this solution is added 1ml of 2% acetyl glucosamine in the form of Chitosan-F in a 2% acetic acid solution per 10ml of pilli suspension. The solution is agitated for 10 minutes. Then 0.5ml of 5M calcium chloride solution is added per 10ml of solution. Whilst the liquid is agitated 1ml of Sodium hydroxide solution is slowly added in drops until a fine white haze appears. Agitation continues for 5 minutes. The suspension is then held at 4°C, and constitutes the aqueous phase of the vaccine.

Production of Solution B

A second liquid comprising the vaccine is prepared as an oily liquid comprising Capric caprilyic triglyceride, polyglycol, polyricinoleate emulsifier and liquid lecithin.

These ingredients are blended in an ultra tarax to produce a uniform liquid. Adjuvants such as heat inactivated Mycobacterium bovis or muramyl di peptide may also be added at this stage.

To prepare the vaccine for injection, 1 part of liquid A is mixed with 2 parts of liquid B and emulsified together in an ultra tarax until a viscous mayonnaise like product of uniform texture results. This can be stored at 4°C until use.

By regulating the concentration of the SRIF and the pilli in the original aqueous liquid (A), the concentration of the SRIF in the final emulsion is adjusted. Ideal concentrations for injection are:

i) 80µg SRIF per ml of final emulsion injected at a rate of 0.1ml per day old chicken,

ii) 500µg SRIF per ml of final emulsion injected at a rate of 1ml per adult sheep or pig.

Results obtained from vaccination of subjects

a) Day old chickens

1. A single subcutaneous injection of 0.1ml of an emulsion prepared as described containing 80µg SRIF per ml into day old broiler chickens resulted in them (n = 50) being 14% heavier at 14 days of age ($P<0.001$) than unvaccinated controls.

2. A single injection of a 50µg per ml SRIF emulsion prepared as above injected intraperitoneally into day old chickens resulted in them (n = 15) being 18% heavier 21 days after injection than chickens vaccinated with the same dose of vaccine intramuscularly at one day old ($P<0.01$).

3. Two groups of 50 day old broiler chickens were randomised at one day old. One group was an unvaccinated control, and the other was injected with 0.1ml of the SRIF vaccine (80µg of SRIF per ml) subcutaneously in the neck at day old and again at 21 days of age.

At 42 days, the average weight of the vaccinated birds was 15% heavier than the unvaccinated birds. No alteration in the relative length of the leg bone to live weight, or weight of abdominal fat pad to live weight at 42 days of age was observed.

4. A confirmatory trial was performed on broiler chickens to confirm that it was the antigen (SRIF) in the delivery system, and not the delivery system per se which was responsible for the growth enhancement.

Vaccinations were performed at day old by injection of 0.1ml of emulsion subcutaneously in the neck of the chickens, or intraperitoneally. At 14 days of age, the following responses were recorded:

TREATMENT	% ADVANTAGE IN BODY WEIGHT GAIN OVER UNVACCINATED CONTROLS
Subcutaneous route	
(a) no antigen	nil
(b) + antigen	5.2
Intraperitoneal route	
(a) no antigen	nil
(b) + antigen	6.2

It is clear from these results as well as from the fact that antibodies to the SRIF delivered can be detected in serum, gut washings, bile and in the white and yolk of eggs that the vaccines according to the invention directly result in growth enhancement. The detection of antibodies in this way suggests that the antibodies are primarily secretory in nature and of the IgA type.

The vaccine has resulted in specific anti-SRIF antibodies which have blocked the natural inhibition to the release of growth hormone, and the other active peptides in the cascade.

Example 9 - Evaluation Of A Milk Replacer Supplement In Protecting Calves Against Clinical Disease When Challenged Orally With Salmonella Dublin.

This example serves to illustrate the efficacy of an orally delivered vaccine comprising passive antibodies against neonatal diarrhoea in calves. The incidence of salmonella infections in dairy cattle and calves is significant in Victorian herds and is of major importance in dairy herds world wide. The disease is currently of major importance in intensively housed calf production sheds in Europe.

Calves were primed with *Salmonella typhimurium* primer on two occasions: on arrival at the pre challenge property, and 4 days prior to challenge. The primer culture comprised 10-12 drops per calf of 10^{-30} of 70% ethanol

extract of *S.typhimurium* (1ml of culture of cell density 2 x 10^{12} cfu/ml was added to 9ml of 70% ethanol, and then diluted by "30 logs").

20 ml of milk replacer supplement was administered daily to the milk supplied to each calf from 4 days prior to challenge until termination of the study (14 days post-challenge). The milk replacer supplement comprised an egg yolk extract derived from eggs from hens vaccinated with 2 x 0.5 ml of formalin inactivated *S.typhimurium* at 8 x 10^{11} cfu/dose at a 14 day interval. The eggs were collected after 10 days. The eggs are cracked open and the yolk removed, The membrane of the yolk is also discarded. The yolk is diluted with sterile distilled water (ideally at a dilution rate of 4 parts water to 1 part yolk). Thorough mixing occurs. The natural fatty substances in the yolk are precipitated out by adding propyl carboxyl methyl cellulose or similar material. To the water/yolk solution, mixing and allowing to stand for 24 hours at room temperature, the substance is recovered. Concentration of the water/yolk solution may occur by the use of reverse osmosis, or other membrane system. The concentrated yolk solution containing the antibodies is then spray dried to yield an *S.typhmurium* rich powder in which the antibodies are coated with the acid protecting polymer. The vaccine administered comprised one part *S.typhimurium* base and two parts slow release acetyl glucosamine base.

Calves were acquired and tagged and the relevant calves pre-treated with 10 drops of Salmonella pre-treatment given orally. They were reared on antibiotic supplemented whole milk (oxytetracycline 3mg/kg/day estimate) and were challenged with 1 x 10^9 cfu/calf *S.dublin* orally as a 1ml drench on day 0 and day 7.

Calves were monitored daily for general health for 16 days post challenge during which time an acute salmonella disease outbreak occurred in the total herd of 52 calves being tested.

The clinical health score below was used to assess the staus of all calves on the specified test dates.

points 1 - loose diarrhoea - yellow grey in colour and indicating mild reaction

2 - watery dark scour and severer than grade 1 diarrhoea

3 - severe projectile enteritis with blood and/or mucous

4 - death

Rectal swabs were taken on days 1,2,4,7 and 11 post initial challenge for presence of challenge organism (to confirm infection had occurred).

The calculation of the severity of clinical symptoms (group data) was made as follows:

(summation of points score) / (recorded days x calf number).

Of the calves vaccinated the score calculated for the severity of clinical symptoms was 12/(9x6) = 0.22. Amongst the control animals the score was 36/(9 x 7) = 0.57.

Under the conditions in which this trial was conducted, the prophylactic regimen afforded the calves gave a marked degree of protection as a group against salmonella disease measured on clinical signs (0.22 compared with 0.57). This test was carried out in conjunction with a test program using a bivalent prototype Salmonella vaccine. The treatment of a combined priming treatment followed by an oral in feed supplement program compared favourably with reduced clinical disease seen in the calves vaccinated with the prototype vaccine.

The vaccination program evaluated represents a more reliable management procedure than the currently available use of antibiotics in milk replacer diets. These latter prophylactic measures create management problems from variability of antibiotic intake, development of antiobiotic resistance in bacteria in the gut flora, and modification of the gut flora by suppression of antibiotic sensitive normal flora organisms.

The microparticles according to the invention are effective not least because they are not inflammatory at the site of attachment to the mucosal wall. By comparison, most, if not all prior art immunostimulants act by creating an inflammatory response thereby increasing exposure of the antigen to the lymphocytic cells. The immune response to the microparticles according to the invention comes from better targeting the antigen to the immune system. This is achieved by the unique method of antigen presentation. This unique method of presentation not only elicits an unexpected immune response, it also

demonstrably interferes with obstructive factors in the gut which tend to limit the activity of antigens previously delivered orally. This is clear from the ability of the microparticles to block endotoxins usually associated with *E. Coli* pili, permitting the antigen to express its full capacity without the effects of a frequently fatal toxin. Thus, rather than just facilitating uptake, the delivery system of this invention influences the ability of cells to process the active agent and illicit an effective response.

It will be appreciated that the delivery vehicle of the invention is uniquely conceived to provide for better immune or biological response, more sustained delivery and particularly oral delivery of the antigen to the relevant immunogenic site.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A biodegradable microparticle comprising one or more bioactive molecules, and a biofilm polymer, said biofilm polymer being of a type such that transport of the bioactive molecule can be effected to the appropriate mucosal surface, and such that slow release of said one or more bioactive molecules is enabled, whereby an effective level of immune or biological response is generated.

2. The microparticle according to claim 1 further comprising a matrix element adapted to render the microparticle of a size suited to uptake at mucosal surfaces.

3. The microparticle according to claim 1 or 2 wherein said biofilm polymer is polysaccharide.

4. The microparticle according to any one of claims 1-3 wherein said biofilm polymer is N-acetyl glucosamine.

5. The microparticle according to any one of claims 2-4 wherein said matrix element is a cell fragment.

6. The microparticle according to any one of claims 1-5 wherein said bioactive molecule is selected from the group consisting of one or more bacterial or viral antigens and natural or synthetic peptide sequences.

7. A vaccine for oral delivery of bioactive molecules to a mucosal surface comprising a microparticle as claimed in any one of claims 1-6 in adjunct with pharmaceutically acceptable carriers and excipients.

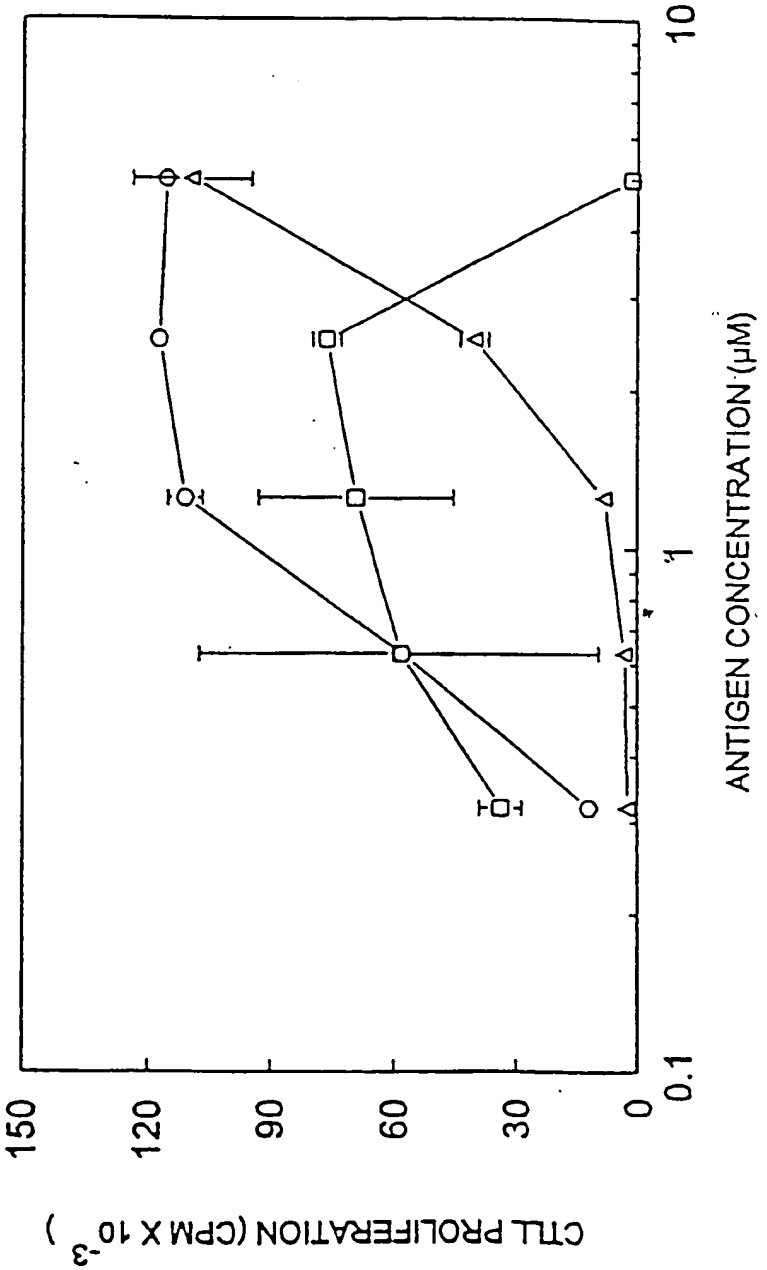
8. A vaccine as claimed in claim 7 wherein said carrier is an oily vehicle.

9. A method of altering physiologic function comprising orally delivering a peptide to a patient requiring treatment in a manner whereby an immune response is stimulated at least partially blocking the activity of the delivered peptide and of equivalent naturally occurring peptide, said orally delivered peptide being encapsulated in a biodegradable microparticle comprising in addition to said peptide, a biofilm polymer.

10. A method as claimed in claim 8 wherein said microparticle additionally includes a matrix element.

11. A method of growth enhancement comprising administering to a patient requiring such treatment an effective amount of a biodegradable microparticle, said microparticle comprising Somatotropin release inhibiting factor, and a biofilm polymer.

FIGURE 2 - T CELL ACTIVATION IS IMPROVED USING THE INOVAX MICROPARTICLE



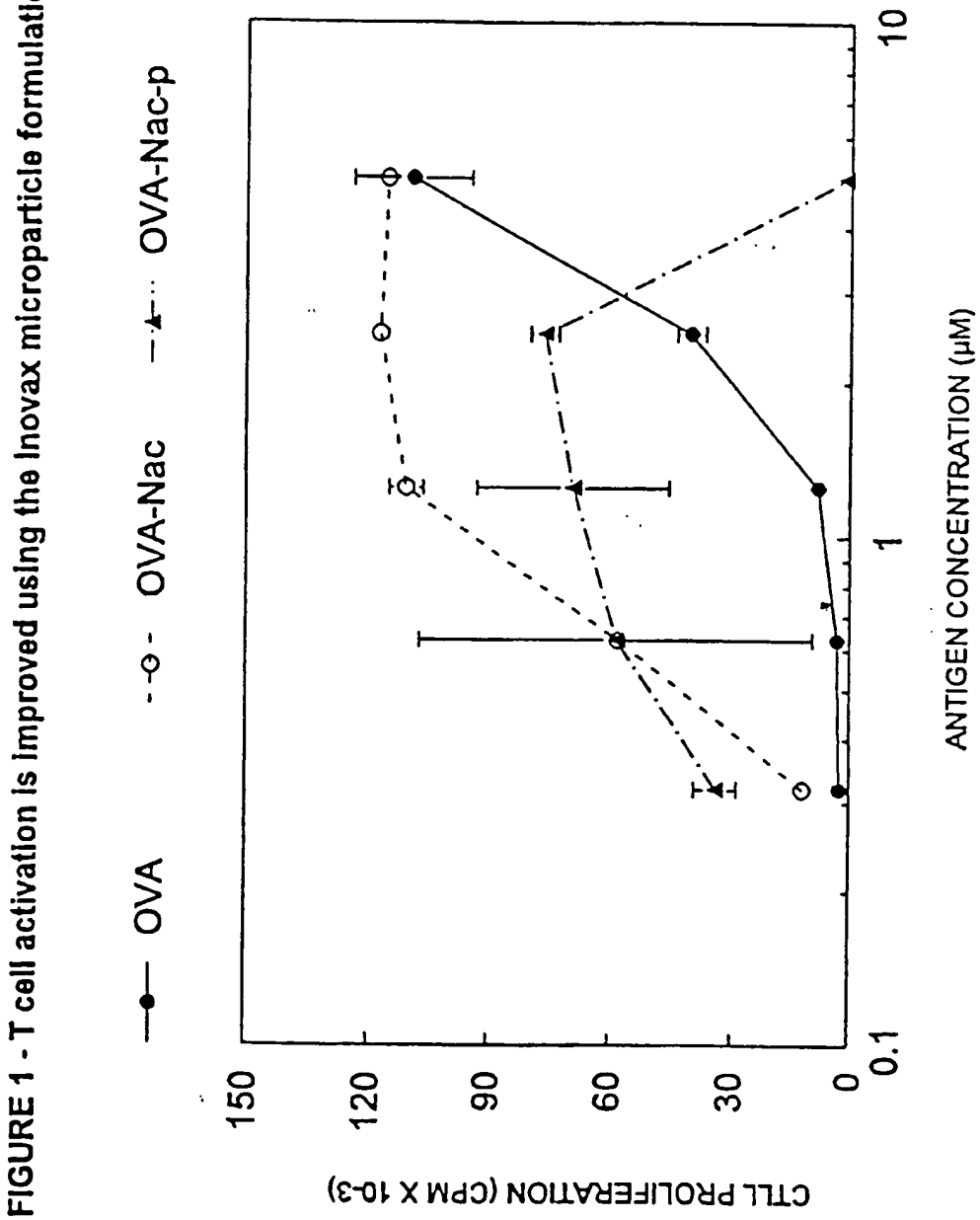


FIGURE 1 - T cell activation is improved using the Inovax microparticle formulation

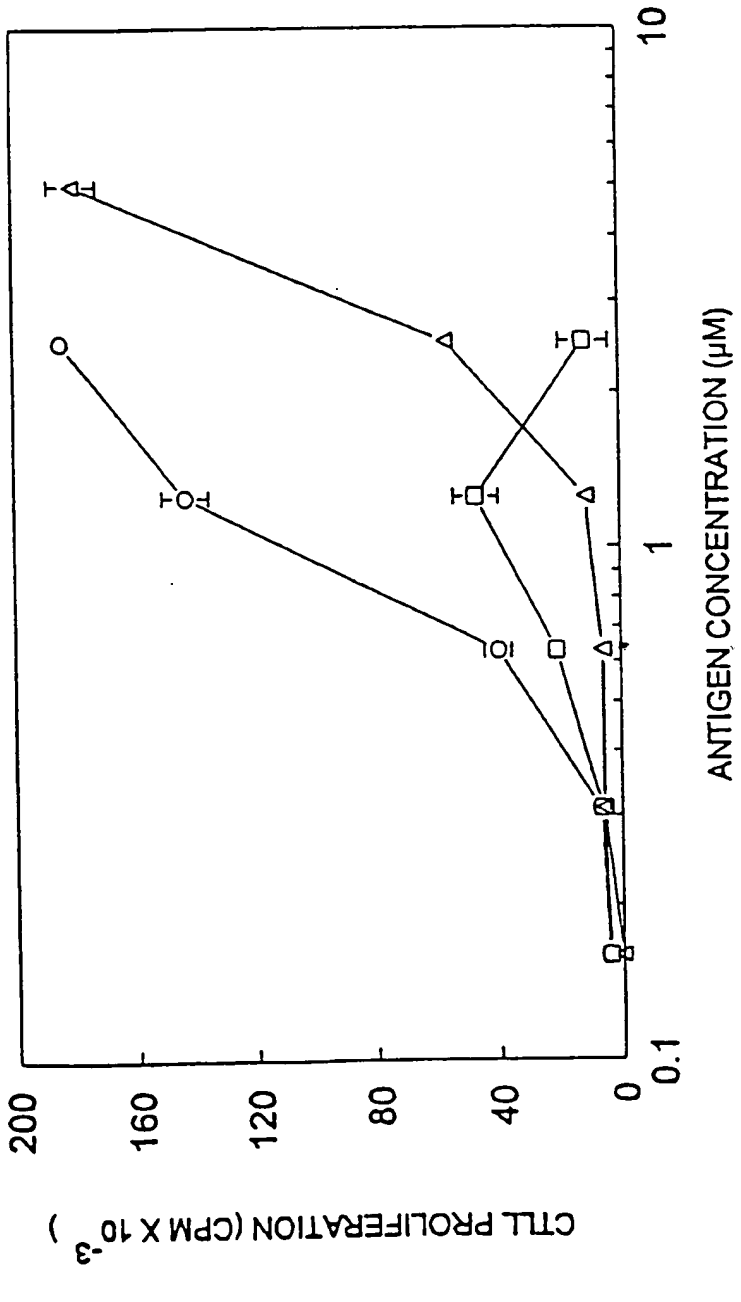
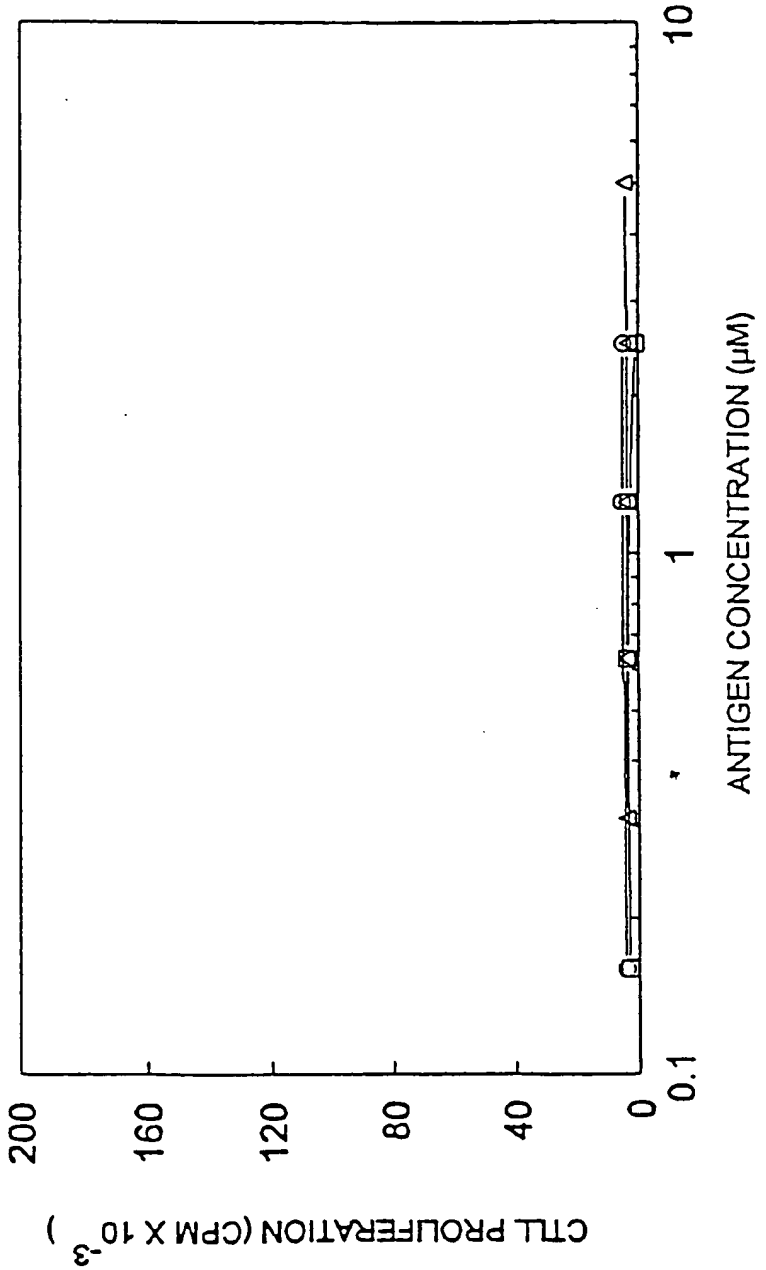


FIGURE 3 - T CELL ACTIVATION IS IMPROVED USING THE INIOVAX MICROPARTICLE (Repeat Experiment)

FIGURE4 - IL-2 PRODUCTION IN ABSENCE OF ANTIGEN-PRESENTING CELLS



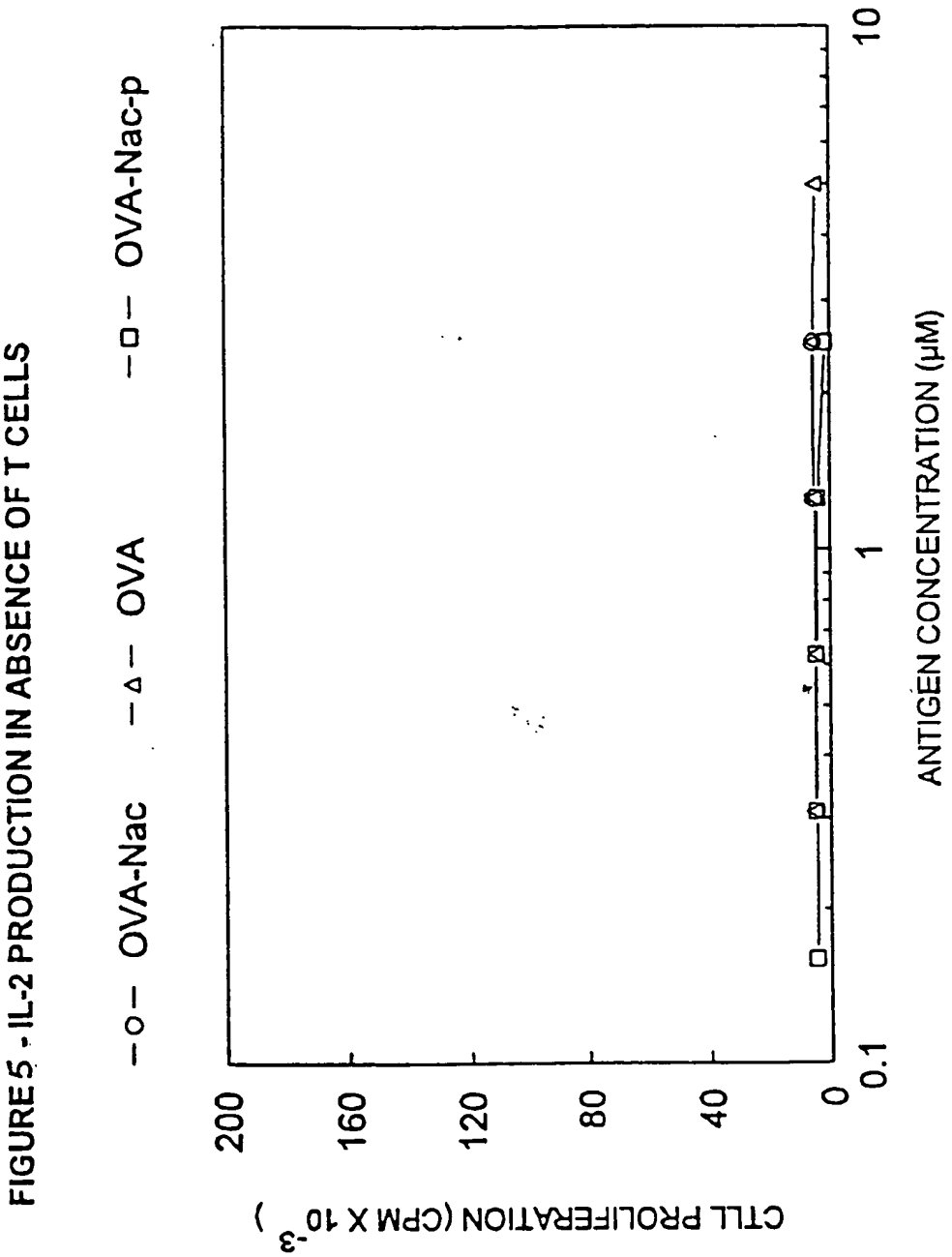


FIGURE 5 - IL-2 PRODUCTION IN ABSENCE OF T CELLS

FIGURE 6 - T CELL ACTIVATION IS IMPROVED WITH INOVAX MICROPARTICLES
(SPLENIC ANTIGEN-PRESENTING CELLS)

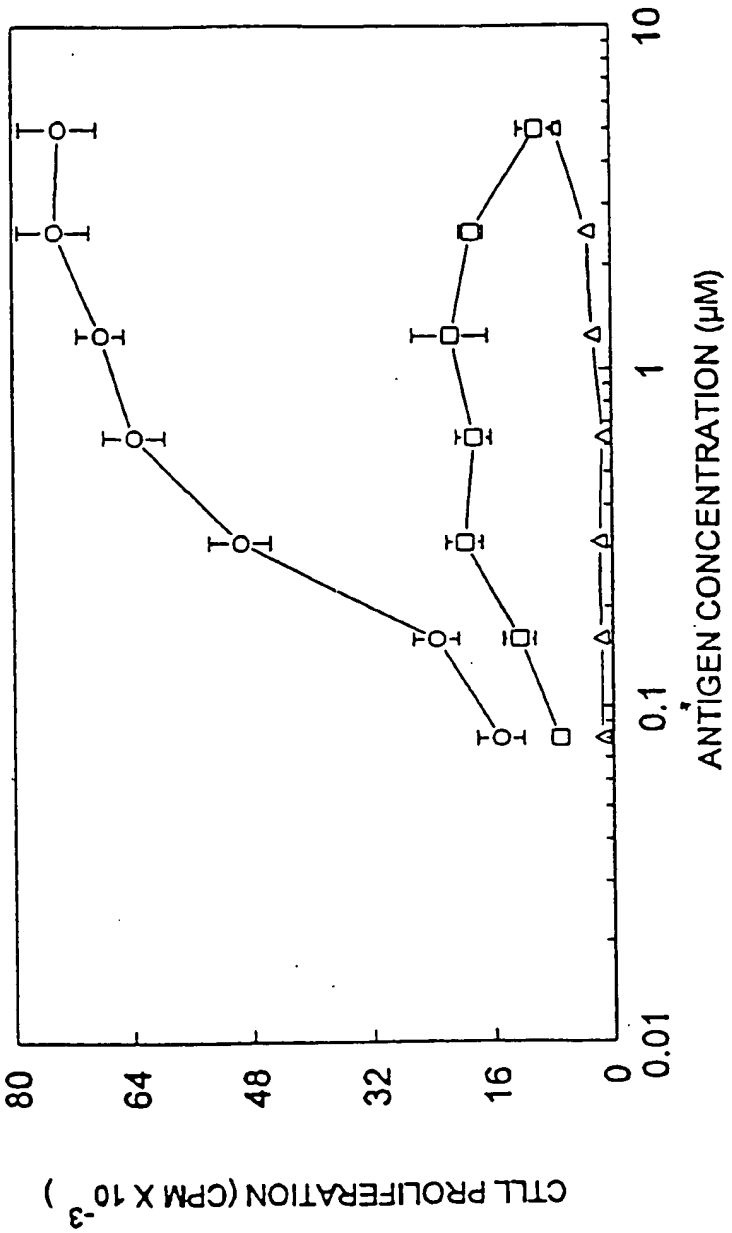
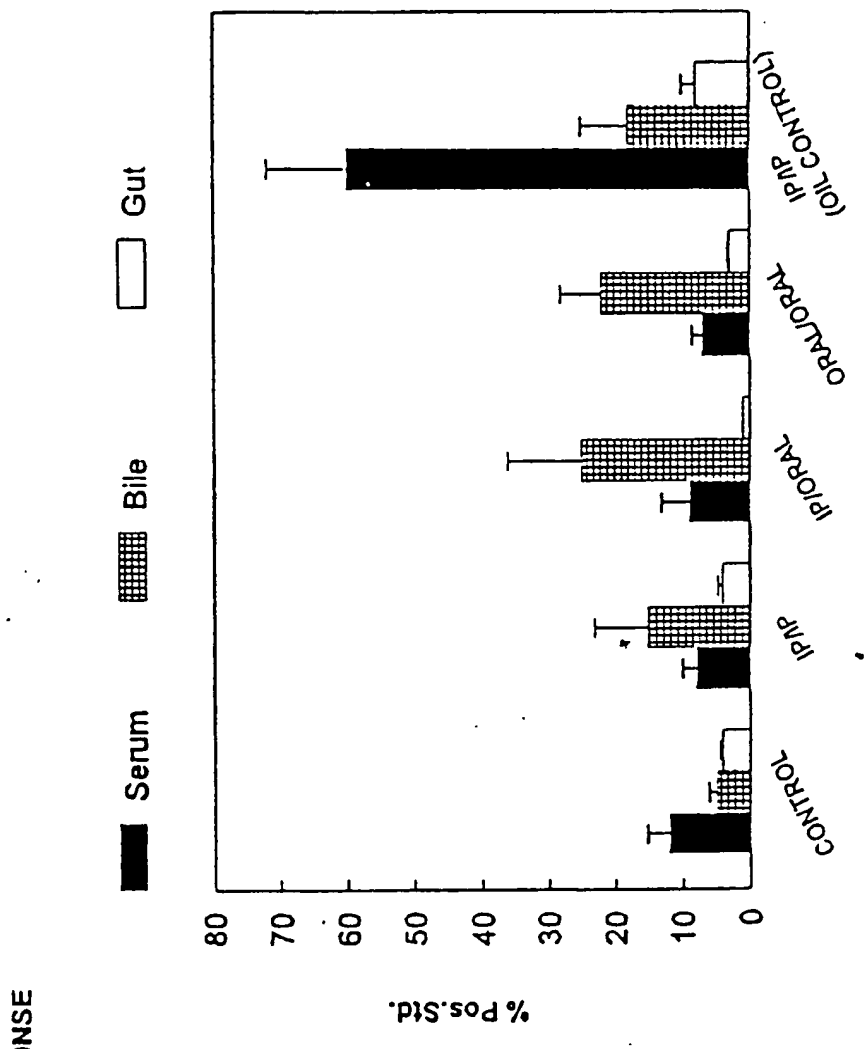
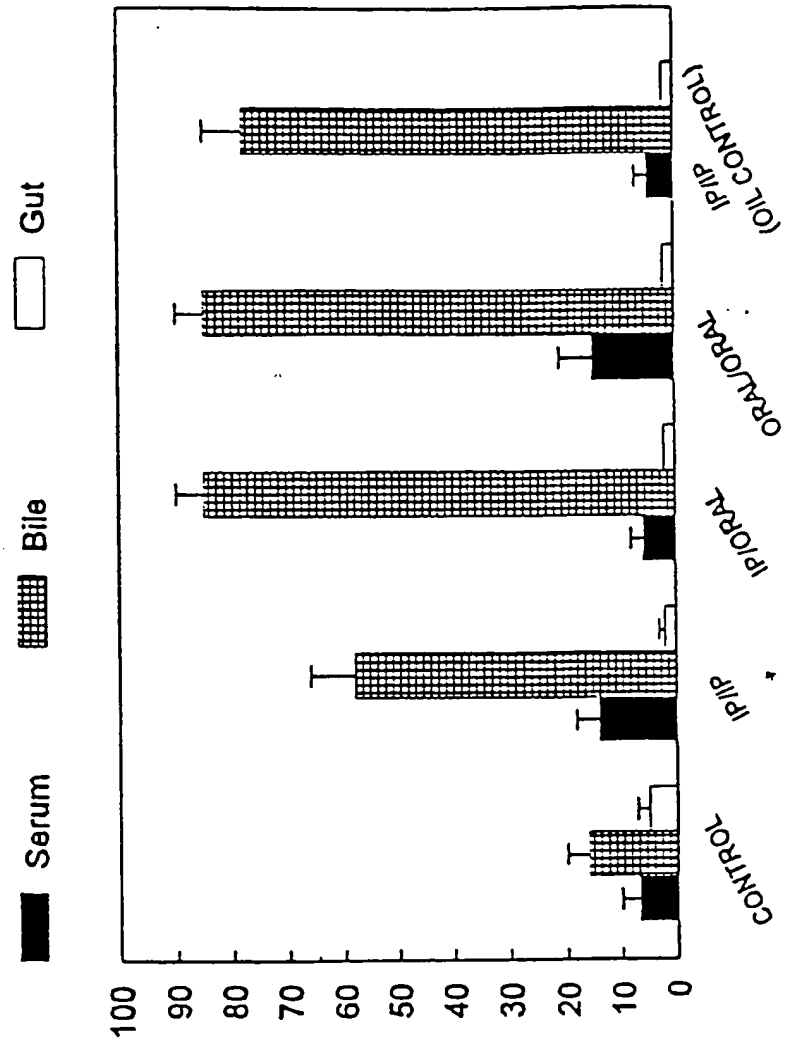
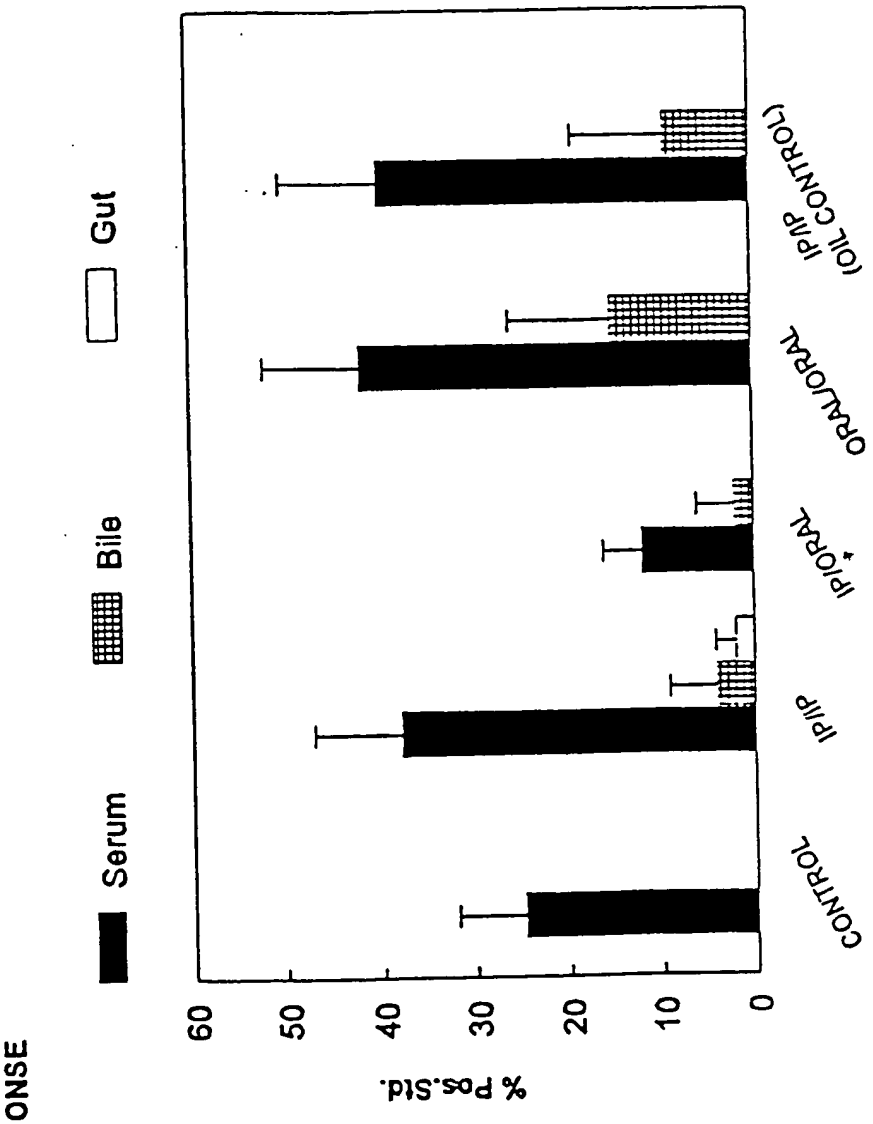


FIGURE 7 - CHICKEN ANTIBODY RESPONSE TO CAMPYLOBACTER FLAGELLIN IN INOVAX MICROPARTICLES





B. IgA RESPONSE



C. IgM RESPONSE

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 95/00291

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.[6] A61K 9/16, 38/18, 39/02, 39/106, 39/112, 39/12, 39/145, 39/17, 47/36, 47/38

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC[6] (A61K 39/- or 38/- or 37/-) and (A61K 47/36 or 47/38)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
WPAT
CASM

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X Y	EP 263490 A1 (CHUGAI SEIYAKU KABUSHIKI KAISHA) 13 April 1988	1-3, 6 7-10
X	EP 454044 A1 (HOECHST AKTIENGESELLSCHAFT) 30 October 1991	1-3, 6-10
X Y	EP 486959 A1 (VECTORPHARMA INTERNATIONAL SPA) 27 May 1992	1-3, 6 7-10

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 July 1995	8 AUGUST 1995 (08.08.95)
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929	Authorized officer GILLIAN JENKINS Telephone No. (06) 2832252

INTERNATIONAL SEARCH REPORT

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X Y	EP 588255 A1 (DOTT LIMITED COMPANY) 23 March 1994	1-3, 6 7-10
X Y	WO 88/07365 A1 (DAVID F RANNEY) 6 October 1988	1-3, 6 7-10
X	WO 90/15596 A1 (ALPHA BETA TECHNOLOGY) 27 December 1990	1-3, 6-10
X	DE 4121891 A1 (FARMITALIA CARLO ERBA SRL) 9 January 1992	1-3, 6
P,X	WO 95/02416 A1 (VIRUS RESEARCH INSTITUTE) 26 January 1995	1-3, 5-10

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member					
EP 263490	AT	116540	AU	79404/87	CA	1315200
	DE	3750943	ES	2068810	JP	63091325
	KR	9307247	US	4853226		
EP 454044	CA	2041093	DE	4013110	JP	4225915
	DE	4035187				
EP 486959	EP	486763	IT	9022155	JP	4283510
EP 588255	AU	46289/93	CA	2106038	CN	1090508
	JP	6199681				
WO 88/07365	AT	90554	AU	16275/88	DE	3881881
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DE 4121891	GB	9014871	GB	9114493	GB	2245831
	IT	1248543	JP	4279530		
WO 95/02416	AU	73261/94	WO	95/02415	WO	95/02628
	AU	73286/94				

END OF ANNEX